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Changes in cellular and molecular functions that contribute to the development and progression of breast cancer are largely undefined. Changes in activities and/or expression levels of components of growth regulatory signal transduction pathways that correlate with tumor progression have been noted. Understanding the functional significance of these biochemical correlates holds the promise for developing new and alternative strategies for prevention, detection, diagnosis and treatment of breast cancer. Protein kinase C (PKC) is a family of ubiquitously expressed enzymes known to be important in regulating basic mechanisms of cell growth and differentiation. PKC is also considered to be a significant signaling pathway in tumor promotion/progression since PKCs are the major cellular receptors for tumor promoting phorbol esters. **The purpose of this proposal is to provide a clear understanding of the role of PKC isozymes and substrates in the transition of a normal breast cell into a cell with altered growth potential and subsequently into a cell with invasive, metastatic potential.**

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INTRODUCTION

Changes in cellular and molecular functions that contribute to the development and progression of breast cancer are largely undefined. Changes in activities and/or expression levels of components of growth regulatory signal transduction pathways that correlate with tumor progression have been noted. Understanding the functional significance of these biochemical correlates holds the promise for developing new and alternative strategies for prevention, detection, diagnosis and treatment of breast cancer. Protein kinase C (PKC) is a family of ubiquitously expressed enzymes known to be important in regulating basic mechanisms of cell growth and differentiation. PKC is also considered to be a significant signaling pathway in tumor promotion/progression since PKCs are the major cellular receptors for tumor promoting phorbol esters. **The purpose of this proposal is to provide a clear understanding of the role of PKC isozymes and substrates in the transition of a normal breast cell into a cell with altered growth potential and subsequently into a cell with invasive, metastatic potential.**

PKC and Mammary Cell Biology. Epidemiological studies have not yet identified a dominant etiology for breast cancer. However, since many of the major risk factors (age at menarche, age at first full-term pregnancy, age at menopause) are linked to natural hormone production, it is likely that endogenous hormone levels play an important role in mammary tumor promotion and progression. There is evidence that PKC is involved in two important aspects of mammary carcinogenesis: estrogen-independence and proliferation.

• *PKC and Estrogens.* In MCF-7 cells, PKC activation was associated with decreased estrogen signaling. Phorbol esters caused down regulation of estrogen receptor message and modulation of AP-1/estrogen receptor interactions (1-3). Overexpression of PKCα in MCF-7 cells decreased estrogen receptor mRNA and estrogen-dependent gene expression (4). PKC has been implicated in the loss of hormonal dependence that accompanies mammary tumor progression since PKC expression was increased in estrogen-independent compared to estrogen-dependent human breast cancer cells (5). Epidemiological studies have indicated a correlation between estrogen-independence and up regulation of EGF signaling pathway components, although a direct role for EGF signaling in developing estrogen-independence has not yet been demonstrated. Since EGF stimulates PKC activity in a variety of cells, PKC is also implicated in regulation of estrogen sensitivity.

• *PKCs and Proliferation.* Several investigators have studied the effects of phorbol esters on growth and differentiation of cultured mammary cell lines. In summary, the reported phorbol ester effects are variable and may depend on individual properties of different cell lines. It is important to note that phorbol esters stimulated proliferation, inhibited differentiation and altered morphological development of undifferentiated, primary cells (6). Since this is the cell population most sensitive to chemically induced mammary carcinogenesis, these results indicate a role for PKC in early events in mammary cell transformation. PKC-dependent effects are likely to be mediated through

a variety of physiological regulators of mammary cell growth and differentiation which have been shown to work at least partially through PKC-dependent pathways:

•*PKC and prolactin.* Prolactin-stimulated growth of cultured mouse mammary epithelial cells is accompanied by increased PKC activity and redistribution of PKCs from soluble to particulate fractions (7).

•*PKC and EGF.* EGF is among the most potent of growth factors for cultured mammary epithelial cells (8). EGF stimulates the activity of PLCγ to produce DAG, the endogenous regulator of PKC activity. A correlation between increased EGF receptor and PLCγ levels in estrogen receptor negative primary human breast cancers has been noted (9), and may suggest that upregulation of this pathway contributes to estrogen-independence of human tumors.

•*PKC and TGFβ.* TGFβ is important for ductal development and differentiation (10). Recent evidence indicates that phosphatidylcholine-specific phospholipase C and PKC may be early intermediates in TGFβ signaling pathways that lead to activation of gene transcription (11).

Taken together, these results indicate an important role for PKC as a downstream regulator and/or effector of mammary growth and differentiation. The role of individual PKCs in these processes has not yet been explored. The overall goal of this IDEA grant is to define the role(s) of PKC signaling pathways in the loss of growth control that accompanies the conversion of preneoplastic to neoplastic lesions and development of metastatic potential in mammary epithelial cells.

Our hypothesis is that PKC isozymes are important for several aspects of mammary tumor promotion and progression including increased growth potential, tumor incidence and invasiveness. **Our goal is to identify the role of individual PKCs and PKC substrates in mammary carcinogenesis using two experimental rodent models.** The first (in collaboration with D. Medina) emphasizes early events in transformation from preneoplastic to neoplastic cells. The second (in collaboration with D. Welch) will help identify PKC signaling events associated with metastatic potential. Our preliminary data demonstrate changes in levels of individual PKCs and PKC substrates in tumors as compared to preneoplasias and in highly metastatic cells compared to less metastatic cells. **The goal of future studies is to test the hypothesis that the observed changes in levels of PKC signaling components drive the transformation and are not merely correlative.** To test this hypothesis, we propose to manipulate the activity levels of individual PKCs in recipient cells by expressing active PKCs or dominant negative PKC constructs. *In vitro* and *in vivo* growth properties of cells overexpressing individual PKCs will be compared. Linking increased activity of an individual PKC to increased mammary cell growth potential, decreased tumor incidence or increased metastatic potential would identify a new therapeutic target for limiting mammary carcinogenesis. Antisense oligonucleotides have proved effective in limiting PKC isozyme expression and function in experimental models (12).

Our preliminary data also indicate that expression of certain PKC substrates is down modulated with progressive transformation. To test the hypothesis that loss of these substrates contributes to loss of growth control, we propose to reintroduce expression of these substrates and determine the effects on cell growth in culture and *in vivo*. These studies will determine if individual PKCs or substrates are suitable targets for developing breast cancer treatment therapies and/or useful markers for diagnosis and prognosis.

BODY

Mammary Tumor Promotion/Progression. **A major goal of the proposed research is to identify biochemical correlates of the transition from preneoplastic to neoplastic growth potential with regard to PKC signaling pathway components.** As with other tissues, the development of mouse mammary tumors occurs through definable stages. The earliest events are associated with a mutation (either inherited or due to unknown exogenous influences) to produce an "initiated" cell with altered growth potential. Additional genetic and epigenetic events produce cells that are increasingly abnormal. The principle limitation of PKC studies in breast has been the limited focus on transformed epithelial models in culture. Such models are not able to consider the impact of stromal-epithelial interactions on growth regulation. The stage-wise progression of neoplastic transformation has been recapitulated in an *in vitro-in vivo* model system of mouse mammary carcinogenesis which has been used to study and identify biochemical correlates of mammary tumor development. Properties of normal, preneoplastic and neoplastic mouse cell populations have been described (13-15). To establish this model, cell lines were generated from primary mouse mammary epithelial cells expanded in three-dimensional collagen gel culture and then passaged into cleared fat pads. After 5 to 10 transplantation generations (TGs), most of the cell lines produced preneoplasias (hyperplastic outgrowths) in recipient fat pads. These TM outgrowth lines were then established as *in vitro* cell lines. Most of the TM lines produce tumors in syngeneic mice; however, some nontumorigenic TM lines have also been isolated. Comparisons of the *in vitro* and *in vivo* properties of the several TM cell lines was used to generate a model for mammary tumor progression.

Normal → Immortalized *(EL11, EL12)* → Hyperplasia I *(TM3, TM2L)* → Hyperplasia II *(TM10, TM12, TM40)* → Hyperplasia III *(TM2H, TM4)* → Metastases *(TM40)*

These lines have been useful for identifying the nature and function of molecular changes associated with mammary carcinogenesis (15,16). Using this model, we have identified reproducible differences in levels of certain PKCs and substrates between hyperplastic lesions and tumors (see below). The combination of the *in vitro-in vivo* approaches provides flexibility in designing mechanistic studies to determine the functional role of identified biochemical changes in tumor development. **To determine if the observed biochemical changes are functional correlates of mammary tumor progression, we propose to prepare cell populations with increased expression**

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of individual PKCs or PKC dominant negative constructs and assess effects of introduction of these genes on growth and tumor incidence *in vivo*.

PKC isozymes in preneoplastic and tumor tissues. As part of studies initiated under my IDEA grant, in collaboration with D. Medina (Baylor), we compared levels of PKC isozymes and substrates in non-tumorigenic hyperplasias, tumorigenic hyperplasias and neoplasias derived from these cell lines. Three types of transformed mammary cell lines were used in these studies: alveolar hyperplasia lines that characteristically produce tumors at a low incidence or a relatively high incidence (TM12 or TM4). TM40 produces relatively few tumors; however, many of these tumors are metastatic.

Levels of PKC isozymes in the preneoplasias **(PrN)** and tumors **(T)** were compared with levels in pregnant glands, which have a comparable level of mitogenic activity. PKC levels were determined by immunoblots stained with PKC isozyme-specific antibodies and quantitated by densitometry **(Table 1)**. The results demonstrate that PKC isozyme contents in pregnant tissue and preneoplastic outgrowths are comparable. However, paired comparisons of levels in tumor tissues to the corresponding preneoplasias revealed significant and reproducible differences. Compared to preneoplastic and pregnant tissue, tumors produced from all five of the TM lines analyzed had increased levels of PKCδ and ζ and decreased levels of PKCε. PKCα levels were not reproducibly changed. Since the pregnant gland, the preneoplasias and tumors are all comprised of proliferating cell populations, it seems likely that the quantitative changes in PKCs observed in tumor tissues are not simply related to differential proliferative states. Furthermore, since increased proliferation has been associated with down modulation of PKC proteins, it is unlikely that the increase in proliferation in the tumors can account for increases in PKCs δ and ζ. **These preliminary results suggest the hypothesis that increased expression of individual PKCs can influence the tumorigenic potential of transformed mammary epithelial cells.**

Table 1. PKC Isozymes in Preneoplastic and Tumor Tissues.

Lesion Type	Cell Line	PKCδ, PrN/T*	PKCε, PrN/T*	PKCζ, PrN/T*
Hyperplasia I	TM3	1.8	0.45	3.5
	TM2L	1.2	0.1	2.4
Hyperplasia II	TM12	6.7	0.2	6.9
Hyperplasia III	TM4	2.9	0.3	3.5
HyperplasiaII/ Metastases	TM40A	2.1	0.3	4.7
Mean $\pm$ S.D.		3.2 $\pm$ 2.5	0.27 $\pm$ 0.2	4.2 $\pm$ 1.7

* PrN, preneoplasia; T, tumor

Mammary Metastatic Progression. Tumor metastasis is the most compelling problem in cancer treatment. Primary tumors can often be effectively treated with surgery and radiation; however, metastatic cells that have colonized other tissues pose an additional threat. In order to spread from the primary sites, malignant cells must invade surrounding normal tissues, penetrate blood and/or lymphatic vessels, dislodge from the primary tumor mass, disseminate to distant sites, arrest in the lymph nodes or microvasculature of distant organs, and finally invade the parenchyma and stroma at these sites to survive and grow into a metastatic colony. It is clear that metastasis is a complex process requiring many biochemical alterations for productive tumor spread. To identify biochemical correlates of the PKC signaling pathway associated with metastatic potential, we have studied PKCs and PKC substrates in clonal cell lines derived from the 13762 rat mammary primary adenocarcinoma or lung metastases (17,18). Clones derived from the primary tumor have low **(L)** or moderate **(M)** metastatic potential (MTC and MTF7, respectively) in the *in vivo* metastasis assay. The highly **(H)** metastatic cells used in these studies were cloned from lung metastatic colonies (MTLn3).

Due to selection during the sequential events required for metastatic growth, the genetic composition of secondary tumors differs from that of primary tumors. Thus, comparison of primary to secondary growths provides an opportunity for defining genetic alterations that are important for metastatic growth. The 13762 rat mammary adenocarcinoma system has been used to isolate metastasis-associated genes by screening subtraction libraries prepared from low and high metastatic clones (19). One of these genes, *mta1*, is a novel sequence that is upregulated in MTLn3 cells and may play a role in breast cancer invasion and metastasis (20). MTLn3 cells also express high levels of the EGF receptor compared to MTC cells (21). Functional significance of this biochemical change was demonstrated by introducing EGF receptor expression into the MTC cells. Increased expression of EGF receptors caused an EGF-dependent increase in adherence to extracellular matrix components *in vitro* and in lung metastases *in vivo*. Thus, expression and ligand-dependent stimulation of EGF receptors appears to be at least in part responsible for the differential metastatic potential of the MTC and MTLn3 cells (21). These results are of potential significance to human breast cancer since there is a negative association between EGF receptor levels and relapse free survival, a positive association between EGF receptor levels and loss of endocrine sensitivity and an increase in EGF receptor levels in breast tumor metastases compared to the primary tumor (summarized in (21)). **We have identified changes in levels of PKC isozymes that may also contribute to the increased metastatic potential of the MTLn3 cells (see below). Future experiments will address the functional significance of these biochemical changes.**

PKC isozymes in progressively metastatic mammary epithelial cells. We have compared PKC isozyme contents in MTC, MTF7 and MTLn3 cells (L, M, H, respectively) in collaboration with D. Welch (Hershey) . PKC levels were compared in high density cultures to minimize potential differences associated with different proliferation rates. Under these conditions, the PKC isozyme profiles of the low and

moderate metastatic cells were comparable. Thus, two independent clones isolated from the primary tumor had similar PKC isozyme profiles. Compared to these cells, PKCδ and ζ levels were increased (1.8 and 2-fold, respectively) in the highly metastatic cells, whereas PKCε levels were decreased. In contrast, PKCα levels did not change. The differences in PKC levels could not readily be attributed to differences in proliferation rates since qualitatively similar results were observed in more rapidly growing, low density cultures. Since the MTLn3 cells were selected *in vivo* for their metastatic potential, it is likely that they have acquired genetic changes relevant for metastatic growth. **The observed biochemical differences in PKC levels suggest the hypothesis that specific changes in PKC isozyme contents may be related to the increased metastatic potential of mammary epithelial cells.** To test this hypothesis, we propose to increase the expression of individual PKCs in the MTC cells and introduce RD dominant negative constructs in MTF7 lines and evaluate how these interventions influence biochemical correlates of metastases in culture and metastatic potential *in vivo*.

PKC Substrates in Mammary Cells and Tissues. We have cloned several PKC substrates according to their high affinity for PKCs in an *in vitro* binding assay (22). Affinity-purified antibodies to the expressed sequences were used to stain immunoblots to compare expression of PKC substrates during mammary carcinogenesis. Levels of two novel sequences, clones 34 and 72, were markedly reduced in tumor *vs* preneoplastic tissues and in highly metastatic *vs* less metastatic cultured cells. In contrast, levels of the PKA binding protein AKAP95 increased. Levels of other PKC binding proteins did not change. Loss of PKC binding proteins/substrates could cause disruption of PKC signaling pathways due to loss of targeting of PKCs and/or due to loss of the functions of these proteins themselves.

CONCLUSIONS

Using two different rodent models for mammary tumor progression, we have found that PKCδ and ζ increase and PKCε decreases as cell become increasingly transformed. In addition, we have found that levels of two novel PKC substrates, clones 34 and 72, decrease with increasing transformation, whereas levels of other PKC substrates do not change. These results strongly suggest the hypothesis that distinct changes in PKCs and PKC substrates are common events in mammary tumor progression and that these changes may be causal rather than correlative. We are prepared to test the functional significance of the observed changes by manipulating the levels of PKCs and substrates in cultured mammary cells and studying the effects of altered PKC and PKC substrate content on mammary cell growth and tumorigenesis *in vivo*.

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